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AGGLUTINATION OF MOTILE SALMONELLAS BY ACRIDINES[1]

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Genetic studies of the determination of Salmonella flagella have suggested that the alternative antigenic phases correspond to two distinct homology groups, i.e., to factors at two distinct genetic loci, one for phase 1 ("specific phase"), and one for phase 2 ("group phase") (Lederberg and Edwards, 1953). It has, however, been generally understood that the two phases were equivalent to one another in all respects save their detailed antigenic structure and thus would give no hint of such a dual determination (Stocker, 1949). Sertic and Boulgakov (1936*a*, *b*) had, however, reported slide agglutination of smooth phase 2 cultures by acriflavine, while phase 1 did not react. We have sought, therefore, to confirm this generally overlooked observation and to look for any further clues that might bear on the reversible differentiation of the two loci which control flagellar phase variation (Lederberg, 1954).

MATERIALS AND METHODS

Various serotypes were selected from stock cultures listed previously (Lederberg and Edwards, 1953). Each was carefully examined for roughness which might interfere with the flagellar reaction. The alternate phases were obtained either by colony selection (Andrewes, 1922) or by selective swarming in motility agar containing absorbed anti-H sera (Edwards and Ewing, N.D.).

Previous investigators have used the slide agglutination technique. Since this method does not lend itself to quantitative titrations and is liable to give false reactions, tube agglutination procedures were preferred. Bacterial suspensions were prepared from overnight cultures in Difco penassay broth inoculated with single colonies of the desired phase and incubated at 37 C. These cultures were diluted to approximately 10^8 cells per ml and used alive, unless otherwise stated.

The agglutinations were conducted in 10 by 75 mm culture tubes held at 37 C and read after 2 to 4 hours. Except for a slight increase in titer, the readings were unchanged after the longer period.

For precipitation tests on isolated flagella, the double diffusion agar gel technique (Ouchterlony, 1949) was chosen after preliminary trials. Flagellar suspensions were isolated from Roccal treated bacteria by method 2 of Uchida, Sunakawa, and Fukumi (1952) from the *b* and *1,2* . . phases of *Salmonella paratyphi* B.

The acridines tested and some analogues are listed in table 3. They were generally made up as 0.2 per cent solutions in distilled water with the addition of dilute acetic acid if required.

RESULTS AND DISCUSSION

The experiments fall into three main groups: those conducted on broth suspensions, those performed with washed aqueous or saline preparations, and those utilizing isolated flagella.

Agglutination of living broth suspensions. The strains were tested in parallel with specific (absorbed) anti-H sera, acriflavine (0.2 per cent in 1 per cent saline), and with Millon's reagent (White, 1929). The results are presented in table 1. Phase reversal was reiterated with several strains, and only phase 2 was agglutinated so long as the strain remained smooth.

It was also found that, unless fully motilized, cells were usually inagglutinable by acriflavine although still capable of reacting with anti-H sera.

Besides confirming Sertic and Boulgakov's

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TABLE 1

Agglutination reaction of strains in broth

Strain	H Antigens	Phase 1				Phase 2			
		Serum		Acridine	Millon	Serum		Acridine	Millon
		Ph. 1	Ph. 2			Ph. 1	Ph. 2		
S. typhimurium	*i:1,2*	+	−	−	S	−	+	+	S
S. stanley	*d:1,2*	+	−	−	S	−	+	+	S
S. zega	*d:z*$_6$	+	−	−	S	−	+	+	S
S. london	*l,v:1,2*	+	−	−	S	−	+	+	S
S. dar-es-salaam	*l,w:e,n,z*$_{18}$	+	−	−	S	−	+	+	S
S. wien	*b:l,w*	+	−	−	S	−	+	−	S
S. abony	*b:e,n,x*	+	−	+	R	−	+	+	R
S. paratyphi B	*b:1,2*	+	−	−	S	−	+	+	S
S. typhi O 901	− [*d*]	−		−	S				
SW666	− [*b*]	−		−	S				
SW926	*1,2:e,n,x*	+	−	+	S	−	+	+	S

Anti-H sera were used at a final dilution of 1 in 1,000. The titers of the sera ranged from 1 in 3,000 to 1 in 300,000. The acridine dye was 0.2 per cent acriflavine.

S and R indicate smooth and rough, respectively, as determined by the Millon reaction (White, 1929). Precipitation alone was taken to indicate roughness.

+ and − indicate presence or absence of agglutination.

findings, these results raise one other point. The antigenic complex *l,w* which appears as phase 1 in some, and as phase 2 in other, serotypic formulae reacts with acriflavine in all cases as if it were a phase 1 complex. The complex *1,2* . . usually represents phase 2 but is present as an anomalous phase 1 in some bacteria (Lederberg and Edwards, 1953). These organisms are agglutinated by acriflavine in both phases. With these exceptions phase 1 homologues are stable and phase 2 homologues are agglutinated by the dye.

Conditions of agglutination in broth. Cells of both phases were washed and suspended in broths which had been adjusted to pH values from 2 to 10 inclusive by the addition of concentrated HCl or NaOH. Both phases agglutinated spontaneously at pH 5; above this value the reaction was unaffected. At pH 4 and below no agglutination of phase 2 cells occurred. If cells exposed to these low pH values were washed and resuspended in normal broth at pH 7, they were still inagglutinable. This suggests that some irreversible change has taken place in the cells during their exposure to these low pH's. It is likely that the flagella are the site of this action since they are known to be acid sensitive (Weibull and Tiselius, 1945).

Organisms heated to 100 C for 15 minutes were no longer agglutinable by either anti-H sera or acriflavine, a correlation which would also implicate the flagella. Furthermore both macroscopically and microscopically the agglutinates resembled those normally formed by anti-H sera.

Since it is inconvenient to work with large volumes of living pathogens, some antiseptics were tested for interference with the dye agglutination reaction. Mercurials and formalin reacted directly with acriflavine to produce a chrome yellow precipitate. If the cells were then washed and resuspended in fresh broth, there was no interference with the reaction. Phenol tended to inhibit the agglutination unless removed by washing. Roccal (benzalkonium chloride, Winthrop-Stearns, Inc., N. Y.) had no effect on the reaction even at bactericidal or higher concentrations, and was used whenever living cells were contraindicated (Anderson *et al.*, 1952).

The effect of broth on the agglutination. The previous experiments were conducted in broth. To determine whether electrolytes were needed, twice washed cells were suspended in distilled water and tested with 0.2 per cent aqueous acriflavine. Contrary to the results in broth all H suspensions were agglutinated equally by the dye, but O cells were unaffected. Apparently this reaction, unlike serum agglutination, does

not require the addition of electrolytes for visible aggregation unless the dye itself (at M/4,000) acts in this respect.

This loss of specificity for the two phases in distilled water might be ascribed to (1) a reagent in the broth, (2) a bacterial metabolic product, or (3) the washing procedure.

Phase 1 cells were washed twice with distilled water, and four aliquots were then resuspended in distilled water, fresh broth, the supernatant broth from the original culture, and the supernatant broth from a phase 2 culture, respectively. Only the aqueous suspension was found to be agglutinable by acriflavine. This supports the hypothesis that normal broth contains an agent capable of inhibiting the agglutination of phase 1 cells by acriflavine. It remains to determine what the agent is and whether the inhibition is absolute or not.

Inhibiting agents. An empiric examination of certain constituents of penassay broth was made to see whether they would inhibit the reaction. Aqueous phase 1 cells were used as indicators. The results are presented in table 2: the purines and pyrimidines in broth could be part at least of the inhibitor. Broth, however, is a very complex medium, and the participation of other components is not excluded.

It was further found that one could overcome the inhibition of any of these agents by adding further acridine. In a titration involving adenine hydrochloride and aminacrine (see below) with aqueous phase 2 cells as indicator, it was found that for every mole of adenine two moles of acridine had to be added to overcome the inhibition. That there is a direct reaction between acridine and inhibitor is suggested, but competition for bacterial sites cannot, on the basis of this evidence alone, be excluded.

TABLE 2

Activity of inhibiting agents

Agent	Dilution	Effect
Saline	40	+
Yeast extract (Difco)	1,600	+
Yeast nucleic acid	3,200	+
Peptone (Difco)	200	+
Tryptone (Difco)	800	−
Guanosine	6,400	+
Xanthine	12,800	+
Thymine	12,800	+
Adenine	12,800	+
Glycine	80	−

The dilution is expressed as the reciprocal of the lowest concentration inhibiting the reaction, or of the highest concentration not causing precipitation of acriflavine in the case of inactive compounds.

+ and − indicate presence or absence of inhibition of acriflavine agglutination.

Titration of acriflavine activity. Doubling dilutions of acriflavine were titrated against suspensions of phase 1, phase 2, and O cells. Preliminary titrations had shown that, unlike serum reactions, the concentration of cells did not, within broad limits, affect the end point.

In broth, O cells were not agglutinated by concentrations up to 1 per cent acriflavine, this being near the limit of solubility of the acridine. Phase 1 cells were agglutinated by 1 in 100 acriflavine, and phase 2 cells were agglutinated at concentrations as low as 1 in 3,000.

In water, the O cells still remained inagglutinable, but the end points for the other two cell types were changed. Phase 1 cells were now agglutinated by 1 in 6,400 and phase 2 cells by 1 in 12,800 final concentrations of acriflavine, respectively.

Activity of other acridines. Several other acridines and some analogues were examined for their effects in the presence and absence of broth. The results are shown in table 3 from which it can be seen that the majority of the acridines behave like acriflavine.

Certain of the reactions are of interest when one considers the theory of acridine bacteriostasis postulated by Albert (1951). He has suggested that a certain "envelope" size is required for the acridine to be effective as an antibacterial agent. Tetrahydroaminacrine would be inactive because the saturation of one of the aromatic rings results in a nonplanar molecule. Similarly, aminoquinoline is too small, but the addition of a styryl side chain increases the envelope to the size required for an active antibacterial agent. The inactivity of the hydroxy compound, on the other hand, was thought to be due to its zwitter-ion structure.

In general the broth reactions agree with this theory; the action of tetrahydroaminacrine on aqueous phase 2 cells is, however, discordant. Whether there is a mechanism of acridine activity on flagella different from that involved in bacteriostasis, or whether the acridine compounds

TABLE 3

Acridine dyes and analogues investigated with their activities

Compound	Formula (numbering according to Albert, 1951)	Suspensions					
		Broth			Water		
		O	Phase 1	Phase 2	O	Phase 1	Phase 2
Acriflavine*	mixture of proflavine and euflavine	−	−	+	−	+	+
Proflavine*	2,8-diaminoacridine	−	−	+	−	+	+
Acridine orange (a)*	2,8-diamino-*bis*-dimethyl-acridine	−	+	+	−	+	+
Acridine orange (b)*	2,8-diamino-*bis*-dimethyl-acridine	−	−	−	−	+	+
Euflavine	2,8-diamino-10-methylacri-dinium hydrochloride	−	−	+	−	+	+
Aminacrine	5-aminoacridine	−	−	+	−	+	+
Tetrahydroaminacrine	1,2,3,4-tetrahydro-5-amino-acridine	−	−	−	−	+	+
Hydroxyaminacrine	2-hydroxy-5-aminoacridine	†	†	†	−	+	+
Aminoquinoline	4-aminoquinoline	−	−	−	−	−	−
Styrylaminoquinoline	4-amino-2-styrylquinoline	†	†	†	−	+	+

* These compounds were obtained from commercial sources and the remainder through the kindness of Dr. A. Albert and Dr. S. D. Rubbo.

+ and − indicate the presence or absence of agglutination.

† Indicates that the compound was precipitated in the presence of broth.

would display different antibacterial activities if examined in buffered saline rather than in broth has not been decided.

The reason for the difference in activity of the two preparations of acridine orange is not certain. These were both commercial preparations and may well have contained impurities or have differed considerably in dye content.

Bactericidal tests with acriflavine and euflavine were conducted on phase 1 and 2 *Salmonella typhimurium* cultures. No significant difference in the rate of killing was found.

Absorption of acriflavine by bacteria. Concentrated suspensions of O, phase 1, and phase 2 cells were added to acriflavine solutions of different concentrations and pH. Roccal treated suspensions had been estimated photometrically so as to contain equivalent amounts of bacterial substance. After a 5 minute exposure at 37 C the cells were removed by centrifugation, washed, and the acriflavine content of both cells and supernates estimated with the Coleman spectrophotometer at 445 mμ.

Spectrophotometry with acridine compounds is liable to give misleading results owing to their high degree of fluorescence (Albert, 1951). However, no marked differences could be found in the amount of dye retained by the different types of cells under identical conditions.

Reactivity of isolated flagella. Tube agglutination tests were performed with aqueous suspensions of phase 1 and phase 2 flagella. Acriflavine at a concentration of 1 in 6,400 agglutinated phase 2 flagella, but a concentration of 1 in 1,600 was needed for the agglutination of phase 1 flagella. It was found that more concentrated flagellar suspensions were needed for agglutination by acriflavine than by specific antisera. This is in accord with the earlier findings on poorly motile cultures prior to the selection of full motility by passage through semisolid agar.

Plate precipitation tests with acriflavine or aminacrine were uniformly negative although similar tests with specific sera gave strong precipitin lines. Similarly when flagella were disaggregated with dilute HCl (Weibull and Tiselius, 1945), they no longer reacted with either serum or acriflavine in tubes. The treated flagella still responded to serum in plate precipitin tests. The merging of precipitin lines showed no difference between the native and treated flagella preparations.

These results confirm the impression gained earlier that the site of acridine action is the flagellum. It is probable that acid treatment gives rise to particles too small to form sedimentable aggregates with acridine but not with sera. This would also explain the lack of acridine precipitin lines since intact flagella would be too large to diffuse through the agar whereas the degradation products would be too small to be sedimented (Gard *et al.*, in press).

These studies have not settled the physicochemical basis of flagellar differentiation. Thus while Sertic and Boulgakov's original observations are confirmed, closer study has shown that the differences between the phases must be considered on a quantitative, not qualitative, basis. However, the genetic differentiation of the phases as the products of distinct loci is paralleled by this difference in agglutinability, whatever its physical foundation.

Acriflavine has been widely used as a criterion of smoothness (Braun and Bonestell, 1947). The present findings reinforced the caution with which this criterion must be interpreted, especially when dealing with Vi+ (Hirsch, 1937) or with motile organisms.

SUMMARY

Sertic and Boulgakov reported that motile Salmonella in flagellar phase 2 were agglutinated by acriflavine. Their observations have been confirmed for the specific conditions they describe.

Motile smooth *Salmonella sp.* are agglutinable by acridine dyes. Phase 2 reacts at distinctly lower dye concentrations than does phase 1. The agglutination resembles the reaction with anti-H serum in appearance and sensitivity to heat.

Examinations of the reaction in penassay broth and in aqueous suspensions show that yeast extract contains agents that inhibit agglutination, probably purines or pyrimidines. Electrolytes, in excess of the dye itself, are not required.

Experiments on isolated flagella indicate that these are the site of the reaction.

REFERENCES

ALBERT, A. 1951 *The acridines; their preparation, physical, chemical, and biological properties, and uses.* E. Arnold, London, England.

ANDERSON, R. E., STEEN, L., MOSS, M. L, AND GROSS, N. H. 1952 Potential infectious hazards of common bacteriological techniques. J. Bacteriol., **64,** 473–481.

ANDREWES, F. W. 1922 Studies on group agglutination. J. Pathol. Bacteriol., **25,** 515–521.

BRAUN, W., AND BONESTELL, A. E. 1947 Independent variation of characteristics in *Brucella abortus* variants and their detection. Am. J. Vet. Research, **8,** 386–390.

EDWARDS, P. R., AND EWING, W. H. N.D. *A manual for enteric bacteriology.* Federal Security Agency, Public Health Service, Communicable Disease Center, Atlanta, Ga.

HIRSCH, W. 1937 The agglutinability by Trypaflavine of *B. typhosus* and its relation to the Vi antigen. J. Pathol. Bacteriol., **44,** 347–355.

LEDERBERG, J. 1954 Phase variation in Salmonella. Rec. Genetics Soc. Amer., **23,** 51.

LEDERBERG, J., AND EDWARDS, P. R. 1953 Serotypic recombination in *Salmonella.* J. Immunol., **71,** 507–515.

OUCHTERLONY, O. 1949 Antigen and antibody reactions in gels. Acta Pathol. Microbiol., **26,** 507–515.

SERTIC, V., AND BOULGAKOV, N-A. 1936*a* Sur la sensibilité des souches d'*Eberthella typhi* au bactériphage, en relation avec les caractères antigénique. Compt. rend. soc. biol., **122,** 35–37.

SERTIC, V., AND BOULGAKOV, N-A. 1936*b* L'agglutination par la Trypaflavine des *Salmonella* de structure antigénique flagellaire non-spécifique. Compt. rend. soc. biol., **123,** 951–952.

STOCKER, B. A. D. 1949 Measurements of rate of mutation of flagellar antigenic phase in *Salmonella typhimurium.* J. Hyg., **47,** 398–413.

UCHIDA, H., SUNAKAWA, S., AND FUKUMI, H. 1952 Studies on the bacterial flagella. I. Methods of purification. Japan. J. Med. Sci., **5,** 351–355.

WEIBULL, C., AND TISELIUS, A. 1945 Acid hydrolysis of bacterial flagellae. Arkiv Kemi, Mineral. Geol., **B20,** No. 3.

WHITE, P. B. 1929 The *Salmonella* group. System of bacteriology. Med. Research Council (Brit.), **4,** 86–158.